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Rapid and Quantitative Determination of Biological Warfare Agent Brucella Abortus CSP-31 Using Surface Plasmon Resonance

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Abstract- A rapid and simple method for the real time monitoring of *Brucella abortus* CSP-31 (*B. abortus* CSP-31) by using Surface Plasmon resonance (SPR) is first time reported. SPR and electrochemical impedance spectroscopy (EIS) were used to characterize the *B. abortus* CSP-31 interaction on carboxymethyldextran modified gold disc with antibody. The EIS data revealed a decrease in impedance after interaction of antigen with antibody due to the effective binding. The present SPR biosensor is able to detect 0.05 pM concentration of *B. abortus* CSP-31 in phosphate buffered saline in less than 10 minute with linearity from 2.0 to 16.0 pM. The equilibrium constant (K_D) and maximum binding capacity of analyte (Bmax) values for *B. abortus* CSP-31 were calculated to be 7.6 pM and 235.94 m°, respectively. This K_D value classifies the antibody as a high affinity one towards the antigen. Moreover, thermodynamic parameter change in Gibb's free energy was calculated and found to be -63.34 kJ/mol. Interference study was also performed with *B. abortus* cell envelope (CE) antigen and a negative angle change was observed with exemplifying specificity for the antigen-antibody interaction of *B. abortus* CSP-31

Keywords- Biological Warfare Agent, SPR, *Brucella Abortus* CSP-31, Impedance, High Affinity Antibody

1. INTRODUCTION

Detection of biological warfare agents (BWAs) is very important one for the past several years because of their easy availability and their ill effects on human beings if these agents are used in an intentional war or during the peace time to create an epidemic. Among the BWAs, Brucellosis is still one of the most common bacterial zoonosis in the world and an important cause for human suffering and economical losses. This disease is caused by several species of the genus *Brucella.* Conventional microbiological methods such as cell culture and susceptibility tests are considered as the most sensitive approaches for identifying BWAs [1-3]. Regrettably, the above mentioned methods are laborious, require considerable expertise, and do not meet the requirements for the rapid and accurate identification of BWAs in less time. Hence, development of detection methodologies for BWAs including *Brucella* is very vital and unavoidable. The following are the detection methodologies used earlier for *Brucella* such as chemiluminescent optical fiber immunosensor detection of *Brucella* cells [4], a novel protocol for identification and differentiation of *Brucella melitensis, B. abortus, B. suis, B. ovis, B. canis, and B. neotomae* suitable for both conventional and real-time PCR systems [5], a fluorescence polarization assay for the detection of serum antibodies to *Brucella abortus* in water [6], an amperometric enzyme-linked immunosensor using resveratrol as the substrates for horseradish peroxidase for *B. melitensis a*ntibody assay [7], a diagnosis process for canine brucellosis by ELISA using an antigen obtained from wild *Brucella canis* [8], a polymerase chain reaction method for detection of *Brucella canis* in vaginal swabs of naturally infected bitches [9], a second generation competitive enzyme immunoassay for detection of bovine antibody to *Brucella abortus* [10], an autoantibody detection method for the rapid diagnosis of brucellosis [11], pattern recognition of bacterial fatty acid profiles generated by direct mass spectrometric analysis of *in-situ* thermal hydrolysis/methylation of whole cells [12] pathogenic bacteria: their detection and differentiation by rapid lipid profiling with a pyrolysis mass spectrometry [13] and solid-phase radioimmunoassay for the detection of immunoglobulins against bovine *Brucella abortus* [14] and bacteria-modified amperometric immunosensor for a *Brucella melitensis* antibody assay [15] were reported. These methods require either labeling of bio-molecules or a substrate for the detection of BWAs. Hence, there is an essential need for developing label-free real time biosensors for the detection of biomolecules directly in biological samples such as in serum, blood, or urine, etc., which can be more sensitive and less time and labor consuming. Certain label free and real-time detection methodologies using surface plasmon resonance (SPR) were reported [16,17]. Our establishment is working for the defenses against biological toxicants and warfare agents [18-22], hence, in the present study we report a label free real time detection SPR method for the BWA *B. abortus* CSP-31 by using a carboxymethyldextran (CM5) modified gold disc for sensing. The experimental parameters those affect SPR angle change such as temperature and pH were conducted and optimized.

Finally, equilibrium constant (K_D), maximum binding capacity of analyte (Bmax) and change in Gibb's free energy (ΔG) value were deduced.

2. EXPERIMENTAL

2.1. Chemicals and Reagents

N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), phosphate buffered saline (PBS), sodium acetate, bovine serum albumin (BSA) and hydrochloric acid (HCl) were obtained from Fluka. Glacial acetic acid, glycine and NaOH were supplied by Sigma-Aldrich. All chemicals and reagents used were of analytical grade and purification was performed wherever necessary before use.

B. abortus CSP-31 antigen, (M.Wt. 31 kDa) and *B. abortus* CE antigen (M.Wt. 65 kDa) were isolated by the trained biologists in our establishment in addition to the raising of polyclonal mice anti-*B. abortus* CSP-31. For SPR measurements, CM5 modified gold disc with a thickness of 50 nm was used and that was purchased from Xantec Bioanalytics (Germany). Different buffer solutions were used in this study depending on pH [10 mM acetate buffer (pH 3.5-5.5), 10 mM phosphate buffered saline (pH 6.0-7.5) and 10 mM glycine-NaOH buffer (pH 8.0-9.0)] for the optimization of pH. All solutions were prepared using water from a Milli-Q system throughout the experiment.

2.2. Instruments

The biomolecular interactions were investigated using a two channel cuvette based electrochemical surface plasmon resonance system (Autolab ESPRIT, Ecochemie B.V., The Netherlands). The outcome of the SPR measurement was automatically monitored using a data acquisition software version 4.3.1. All kinetic data were obtained using kinetic evaluation software version 5.0 (Ecochemie B.V.). EIS studies were performed with Autolab PGSTAT-302N digital potentiostat/galvanostat using FRAII module with FRA software 4.9 (Ecochemie B.V.). The EIS analyses were conducted within the frequency range of 0.1-10 kHz at open circuit potential. The pH of the buffers was measured with a EUTECH instrument pH meter (pH-1500, Singapore). All experiments were carried out at 25^{o}C unless otherwise stated and the temperature of cuvette was controlled by a Julabo HE-4 (Germany) water bath.

2.3. Immobilization of antibody on CM modified gold SPR sensor chip

To achieve high sensitivity and excellent repeatability in SPR measurements, it is very vital to find out suitable linker reagents those provide strong link between the modified gold disc and the immobilized antibody**.** In order to accomplish the above, immobilization of antibody on the modified sensor chip was performed utilizing EDC/NHS chemistry. Prior to the immobilization of antibody, 50 μL of 10 mM acetate buffer (pH 5.2) was passed every

120s interval for 600 s in order to get a stable baseline in both the channel. First, CM5 gold disc was activated by injecting a 50µl solution consisting of freshly prepared 1:1 mixture of EDC (400 mM) and NHS (100 mM) in distilled water over the chip surface for a time period of 300s in order to get amine reactive NHS esters. This was followed immediately by injection of 50 µl of *B. abortus* CSP-31 antibody (1:1000 dilution [1 µL *B. abortus* CSP-31 antibody in 999 µL of 10 mM acetate buffer]) in channel 2 for 900 s to get an effective immobilization of *B. abortus* CSP-31 antibody on the activated dextran modified surface. 10 mM acetate buffer was injected in channel 1 for immobilization and used as reference channel. In order to improve the sensitivity of SPR measurement, blocking of free activated reactive sites on the sensor chip is warranted to avert non specific adsorption of biomolecules from the sample on sensor surface, to attain the above, after the immobilization of antibody and subsequent washing, 1 M ethanolamine 50 µL was used as the blocking agent in both channel and allowed 600 s to react with the sensing surface. After the blocking, regeneration of the modified sensor surface was achieved by the consequent addition of 0.01 M HCl.

2.4. Biosensing Protocol

The entire SPR sensing was carried out with *B. abortus* CSP-31 antigen between. 2.0 pM and 16.0 pM in order to develop a methodology for the detection of *B. abortus* CSP-31 antigen.

2.5. Optimization of experimental parameters

Optimal temperature and pH for binding of antigen on immobilized antibody were explored by varying the temperature (16-31^{o}C with a 3^{o}C increment) and using different buffers in the pH range from 3.5 to 9.0 during the interaction.

2.6. Interference study

In order to know the specificity of the developed SPR based sensing method for *B. abortus* CSP-31, interference study was conducted at optimized experimental conditions with *B. abortus* CE at different concentrations ranging from 3.0 nM to 300 nM.

3. RESULTS AND DISCUSSION

3.1. Modification of CM modified gold SPR sensor chip with B. abortus CSP-3 antibody

The SPR sensorgram obtained for the immobilization of *B. abortus* CSP-31 antibody is depicted as Fig. 1. In order to get good immobilization of antibody on the SPR sensor chip, nine steps were conducted one after another consecutively as indicated in Fig. 1. It is observed from Fig. 1 that a net angle change of 240 m° is observed by subtracting step one and step nine and this angle change is equivalent to the attachment of 2.0 ng/mm^2 of antibody on the sensor surface [23].

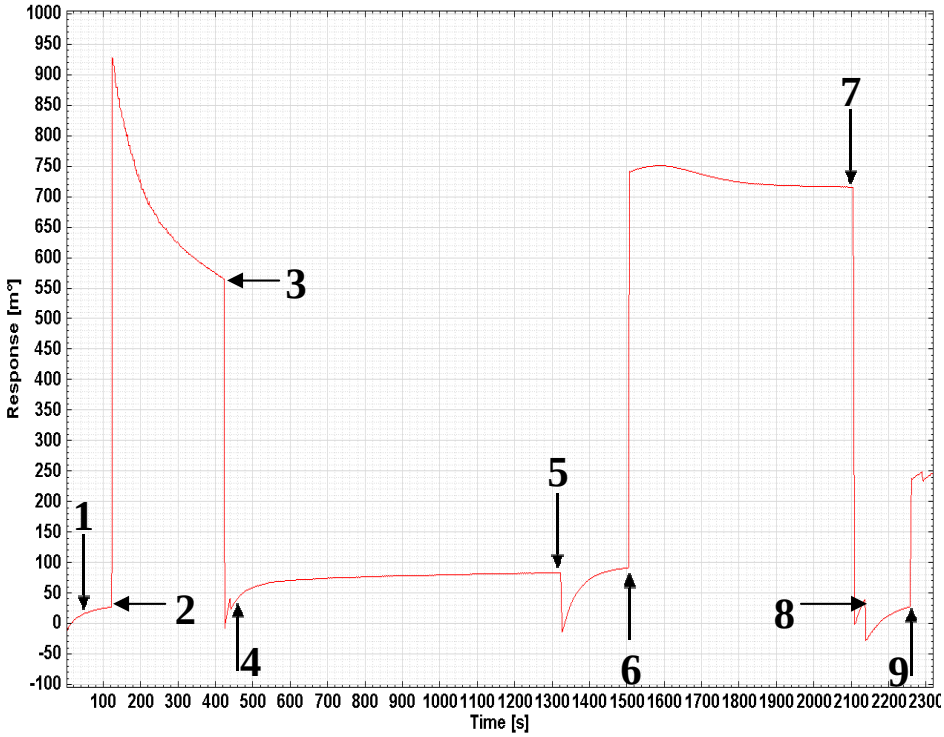

Fig. 1. Sensorgram showing different steps [(1) Baseline (2) EDC-NHS activation (3) Washing (4) Antibody coupling (5) Washing (6) Deactivation (7) Washing (8) Regeneration and (9) Back to baseline] involved in the immobilization of *B. abortus* CSP-31 antibody (1: 1000 dilution) on carboxymethyldextran modified gold disc

3.2. Interaction of B. abortus CSP-31 antigen with the B. abortus CSP-31 antibody immobilized sensor chip

The *B. abortus* CSP-31 antibody immobilized sensor chip was utilized for the sensing of *B. abortus* CSP-31 antigen by interacting with different concentration of the antigen and the results are indicated as SPR sensorgram in Fig. 2. It is known from Fig. 2 that an increase in SPR angle change is observed with increase in the concentration of *B. abortus* CSP-31 antigen from 2.0 pM to 16.0 pM and this observation indicates an effective interaction between the antigen and antibody, thereby gives an opportunity for the real time label free sensing of *B. abortus* CSP-31 antigen. The calibration graph constructed using the data of association phase of Fig. 2A is shown as Fig. 2B which is having linearity between 2.0 and 16.0 pM with a linear regression of 0.99.

Limit of detection (LOD) of the present method was calculated experimentally and was found to be 0.05 pM and this is the minimum concentration of *B. abortus* CSP-31 antigen which showed the response during the interaction of *B. abortus* CSP-31 antigen with its immobilized antibody.

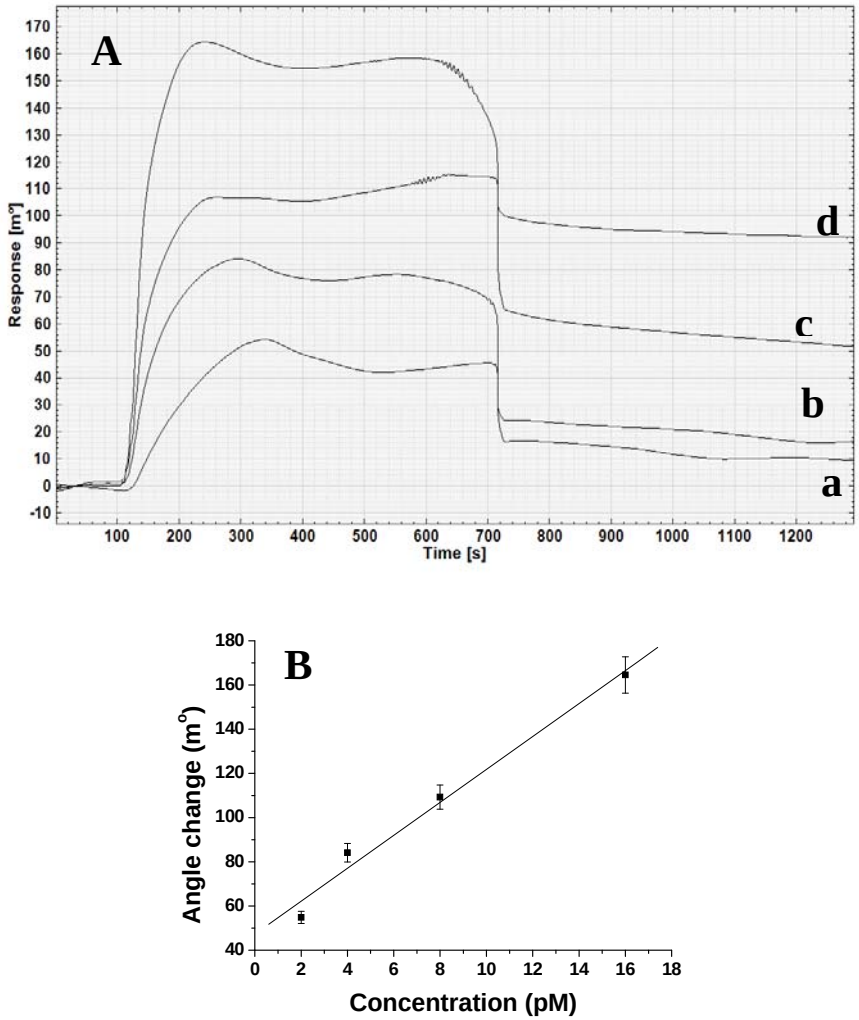

Fig. 2. (A) SPR sensor response for the interaction of different concentration of *B. abortus* CSP-31 antigen with immobilized antibody (a) 2.0 pM, (b) 4.0 pM, (c) 8.0 pM and (d) 16.0 pM and (B) Calibration plot for the interaction of *B. abortus* CSP-31 antigen with immobilized antibody

3.3. Evaluation of kinetics involved in the antigen and antibody interaction

The affinity interactions of *B. abortus* CSP-31 antigen with immobilized antibody were characterized by the equilibrium constant (K_D). The data were fitted using a simple 1:1 interaction model [24], A+B=AB, where 'A' is the injected analyte, 'B' is the immobilized ligand and 'AB' is the analyte–ligand complex formed during the reaction. In the SPR system, the signal 'R' is proportional to the amount of [AB] and the Rmax is proportional to the initial [B].

In this study the kinetics of antigen and antibody interaction was calculated using kinetic evaluation software as discussed in the experimental part. Kinetic parameters such as K_D and

B_{max} value were calculated using the data of the association phase of sensorgram at 600 s and found to be 7.6 pM and 235.94 m^o, respectively. This low K_D value represents high affinity interaction between the antigen and immobilized antibody [25].

3.4. Evaluation of thermodynamic parameter

The thermodynamic parameter, ΔG was evaluated by using Van't Hoff [26] equation and found to be -63.34 kJ/mol and this negative value attributes the spontaneous nature of *B. abortus* CSP-31 antigen interaction with its immobilized antibody on sensor chip.

3.5. Effect of temperature on SPR sensing

Temperature variation study was performed so as to find out the effect of temperature on antigen and antibody interaction from 16 to 31 ^oC as discussed in the experimental part and the results are presented as Fig. 3a. It is clearly inferred from Fig. 3a that the maximum angle change is observed at 25 ^oC. Hence, one can conclude that temperature of 25 ^oC as optimum one to have maximum interaction of antigen with antibody. Therefore, SPR measurements were performed at 25 ^oC in this study in order to get maximum response for the sensing of *B. abortus* CSP-31.

3.6. Effect of pH on SPR sensing

Fig.3b. shows the effect of pH on SPR angle due to the antigen antibody interaction on the carboxymethyldextran modified gold disc. The experiments are conducted with 16.0 pM *B. abortus* CSP-31 antigen with different pH buffer as discussed in the experimental part. It is observed from Fig. 4b. That SPR angle increases as increase in pH up to 7.5 and then started to decrease up to pH 9.0. This observation is probably due to the pH dependent structural changes and electrostatic interactions on the sensor chip between antibody and antigen as reported earlier [27]. All these observations collectively suggests that in pH 7.5 phosphate buffer, *B. abortus* CSP-31 antigen and antibody interaction is as more effective and thereby resulted in more angle change, hence, pH 7.5 was preferred for further studies.

3.7. Electrochemical impedance spectroscopic monitoring of antigen-antibody interaction

Electrochemical impedance spectroscopic study gives information on the impedance change of the electrode/solution or modified SPR disc/solution interface. The impedance can be presented as the sum of the real Z' (ω) and imaginary Z" (ω) components that originate mainly from the resistance and capacitance of the cell and this is known as Nyquist plot [28]. In Fig. 4 the semicircle diameter at higher frequency corresponds to the electron transfer limited process (Ret). Fig. 4a shows the AC impedance spectrum for carboxymethyldextran modified gold disc, Fig.4b is AC impedance spectrum for modified gold disc immobilized with *B. abortus* CSP-31 antibody and Fig. 4c is AC impedance spectrum for *B. abortus* CSP-31 antigen during interaction with its immobilized antibody.

Ret is most influential and direct parameter to reflect the *in-situ* changes those are occurring on the modified disc/electrolyte interface, hence, fit and simulation method was adopted to find out the Ret value for carboxymethyldextran modified gold disc and antibody immobilized SPR sensor chip before and after antigen interaction, the Ret values are found to be 1.45 KΩ, 69.9 Ω and 65.6 Ω, respectively. The decrease in Ret value after the interaction of antigen with antibody directly confirms an increase in electron transfer due to the effective binding of antigen with antibody because of good interaction at this pH (7.5) as reported earlier for antigen antibody interaction [29].

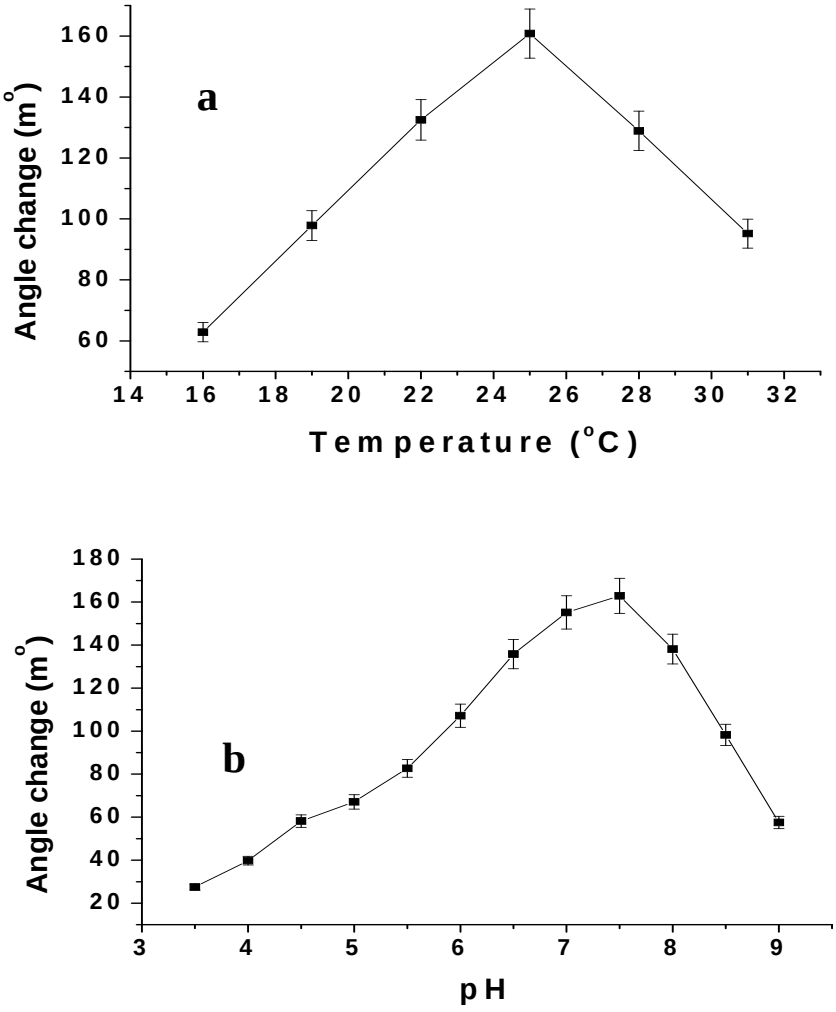

Fig.3. (a) Effect of temperature for the interaction of *B. abortus* CSP-31 antigen with the immobilized *B. abortus* CSP-31 antibody. Temperature range: 16 ^{o}C-31 ^{o}C, pH: 7.5 and concentration: 16.0 pM and (b) Effect of pH for the interaction of *B. abortus* CSP-31 antigen with immobilized *B. abortus* CSP-31 antibody. pH range: 3.5-9.0, temperature: 25^{o}C and concentration: 16.0 pM

3.8. Interference study

In order to know the specificity of the developed SPR based sensing method for *B. abortus* CSP-31, interference study was conducted with *B. abortus* CE concentrations of 3.0, 30.0 and 300.0 nM as depicted in Fig. 5. The negative angle change may be owing to the ineffective interaction between the antigen and antibody [30], thereby no interference is observed for *B. abortus* CE with *B. abortus* CSP-31 antibody and this outcome confirms the interaction between *B. abortus* CSP-31 antibody and *B. abortus* CSP-31 antigen as more specific one.

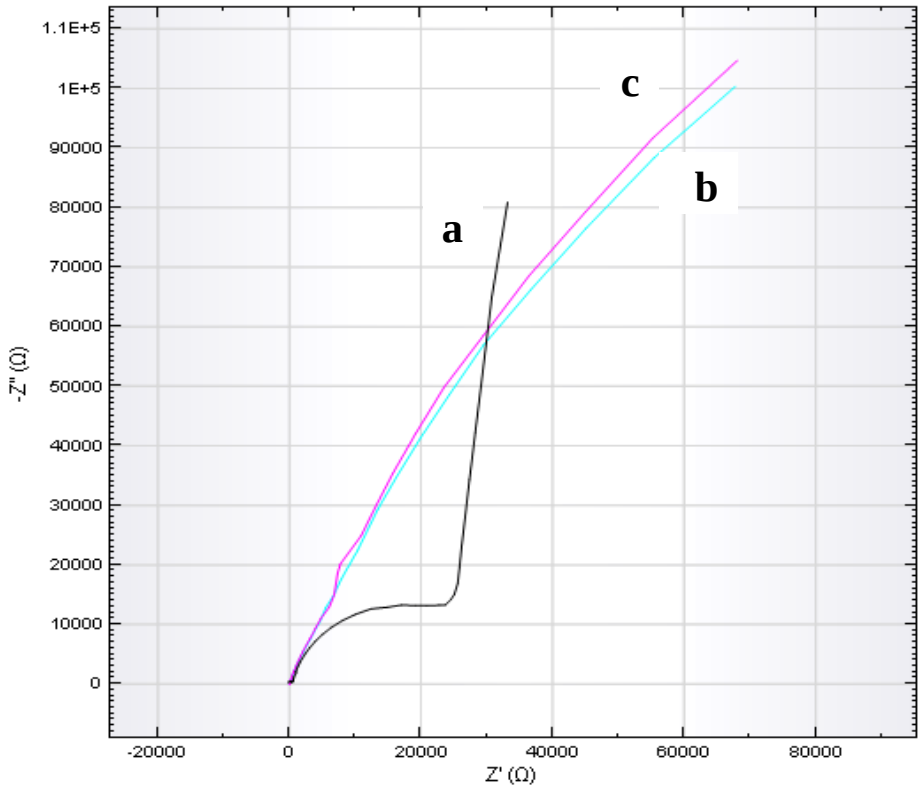

Fig. 4. Electrochemical impedance spectrum (EIS) for (a) carboxymethyldextran modified gold disc (b) modified gold disc immobilized with *B. abortus* CSP-31 and (c) *B. abortus* CSP-31 antigen interaction with immobilized antibody. pH 7.5, temperature: 25^{o}C and concentration: 16.0 pM

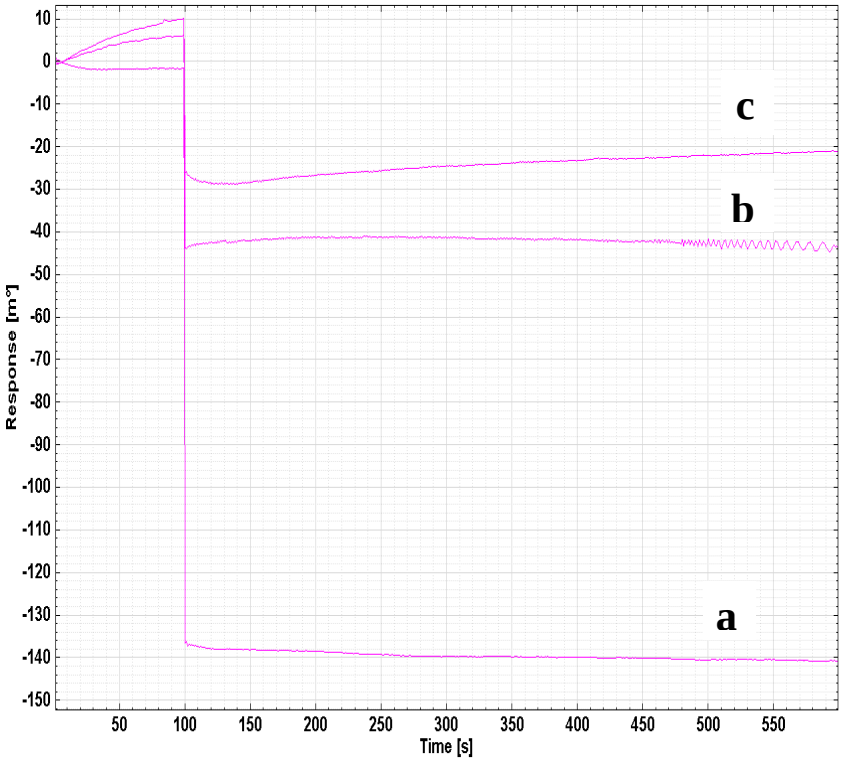

Fig. 5. SPR sensor response for the interference of different concentration (a) 3.0 nM, (b) 30.0 nM and (c) 300.0 nM of *B. abortus* CE antigen with immobilized *B. abortus* CSP-31 antibody

4. CONCLUSION

A label free real time SPR detection methodology for *B. abortus* CSP-31 was first time developed using a carboxymethyldextran modified sensor chip. The EIS characterization data revealed a decrease in impedance after interaction of antigen with its immobilized antibody due to the effective binding. The developed SPR biosensor showed linearity from 2.0 to 16.0 pM with a detection limit of 0.05 pM. The detection time for *B. abortus* CSP-31 by SPR is found to be less than 10 minute. The K_D, Bmax and ΔG value for *B. abortus* CSP-31 were calculated and found to be 7.6 pM, 235.94 m°and -63.34 kJ/mol, respectively. The K_D value 7.4 pM implies and classifies this antibody as a high affinity one. The ΔG value attributes the spontaneous nature of *B. abortus* CSP-31 antigen interaction with its immobilized antibody. Moreover, interference study performed with *B. abortus* CE antigen exhibited a negative in angle change with exemplifying specificity for antigen-antibody interaction. This study gives inputs for the development of SPR based sensors using antigen and antibody interaction with high specificity for *B. abortus* CSP-31 and also for other BWAs.

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